

INVESTIGATION OF THE BIOLOGICAL CHARACTERISTICS
OF AMANTADINE-RESISTANT INFLUENZA A VIRUS

A Thesis

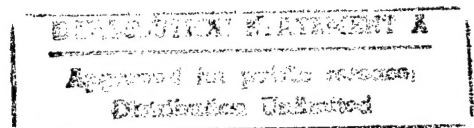
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CAROL CHRISTINE WALTERS

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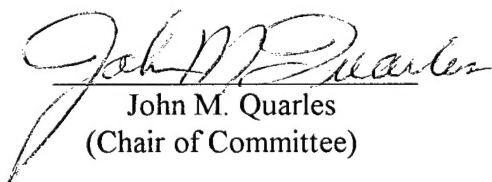
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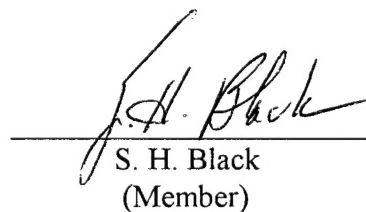
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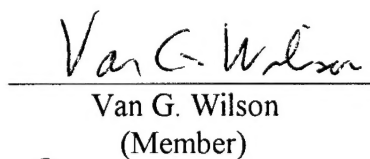
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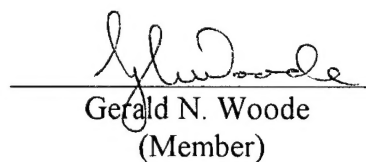
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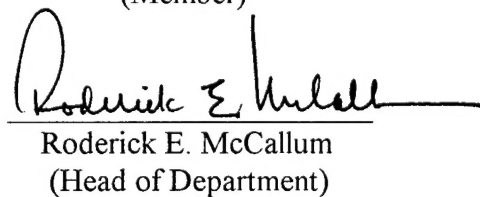
Approved as to style and content by:


John M. Quarles
(Chair of Committee)


S. H. Black
(Member)


Van G. Wilson
(Member)


Gerald N. Woode
(Member)


Roderick E. McCallum
(Head of Department)

August 1997

Major Subject: Medical Sciences

ABSTRACT

Investigation of the Biological Characteristics
of Amantadine-Resistant Influenza A Virus. (August 1997)

Carol Christine Walters, B.S., Texas A&M University

Chair of Advisory Committee: Dr. John M. Quarles

Since the first report of amantadine-resistant strains of influenza in 1981 there has been much speculation about the epidemiological impact of these strains should the use of amantadine and rimantadine become widespread during an influenza pandemic. To date, there have been only two reports of confirmed resistant strains that were isolated from patients with no drug treatment. All other resistant strains have been collected only after therapy with either rimantadine or amantadine.

Because naturally-occurring resistant strains are not isolated more often in the absence of drug therapy, it has been suggested that the drug-resistant phenotype does not confer any type of selective or replicative advantage over the sensitive phenotype. As a corollary, the suggestion was made that those viruses with the susceptible phenotype may have a biological advantage over their resistant counterparts.

This study was conducted to determine if one phenotype of influenza A virus has an advantage in replication over the other. To accomplish this, 30-hour growth curves were generated for one amantadine-sensitive and two resistant virus isolates. Evaluation of the experimental results included visual comparison and numerical analysis of the data.

This study also tested the hypothesis that amantadine alters the environment of the infected cell in such a way as to enhance replication of drug-resistant strains. In order to test this hypothesis, 30-hour growth curves in the presence of 5 μ g/ml were completed for two resistant isolates. Visual and quantitative analysis was used to compare these growth curves to those curves generated for the resistant isolates without the addition of amantadine.

The data presented suggest that drug-resistance or sensitivity alone does not confer any type of replicative advantage. However, it is apparent that within the heterogeneous population of viruses collected from one patient, there are resistant isolates that can achieve maximum titers equal to or greater than those of drug-sensitive strains. These data also provide strong evidence that drug therapy does not enhance or diminish the replicative characteristics of drug-resistant strains.

DEDICATION

To my husband, Brett, for making it through these crazy 18 months.

ACKNOWLEDGMENTS

My sincerest thanks is extended to my committee members, Drs. John Quarles, S.H. Black, Van Wilson, and Gerald Woode, for their advice, comments, and support. I am especially grateful to Drs. Quarles and Black for providing so many extra opportunities to participate in graduate and medical student education.

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INTRODUCTION

The influenza A virus is a medium-sized (80-120 nm), generally spherical virus that can also assume a filamentous form. The virus has a lipid bilayer envelope that is host cell-derived and contains the viral hemagglutinin (HA), neuraminidase (NA), and M2 proteins (35). Lining the cytoplasmic side of the envelope is the M1 protein, which is in contact with the viral membrane/HA and the nucleoproteins that coat the viral genome (14). The genome itself is composed of eight negative-strand RNA segments, each forming a helical structure with its associated nucleoprotein (ribonucleoprotein, or, RNP). At the ends of the RNPs are complexes of three viral polymerases -- PB1, PB2, and PA. Two other proteins, NS1 and NS2, are coded for by the virus, but are not included in the virion. Although their function is currently uncertain, they appear to participate in viral replication (35).

Influenza A enters the host cell via interactions between its HA molecules and the terminal sialic acid of host-cell surface glycoproteins or glycolipids. Once this binding has occurred, the virion is endocytosed by the cell. In the endosome, the HA undergoes a pH-induced conformational change, resulting in the fusion of the viral membrane with that of the endosome (35). At this point, the viral contents are released into the cytoplasm of the host cell where they complete transcription, replication of the viral genome, and construction of progeny virus. These new virions then bud from the host cell and are available to infect neighboring cells.

This thesis follows the style and format of the Journal of Virology.

At this time, only two drugs have been approved for treating influenza A infections. Amantadine HCl was approved in the United States in 1966 for prophylaxis and treatment of H2N2 influenza A infections. In 1976, it was approved for such use in all influenza A infections (15). Amantadine is not effective in treating influenza B or C infections. Rimantadine HCl, an analog of amantadine, is also approved for use in influenza A infections. Although rimantadine is reported to have fewer side effects than amantadine and is more widely used in the former Soviet Union, its use in the United States has been limited (10).

Investigations of Amantadine's Mode of Action

The use of 1-adamantanamine (amantadine) as an antiviral agent against influenza infections was first reported in 1964 (6). This study involved testing the use of the water soluble salt form of the compound, amantadine HCl. According to Davies, the drug produced antiviral effects in tissue culture, chick embryos, and mice. Because Davies was randomly screening drugs for their antiviral activity, he did not know the mode of action of amantadine against influenza A. His study did indicate that the virus was not directly inactivated by amantadine, leading Davies to propose that it blocked or slowed penetration into the host cell.

Hoffmann et al. (17) provided additional evidence that supported Davies' proposal. By infecting chick-embryo fibroblasts and varying the time at which amantadine was added, they determined that amantadine interfered with virus replication when added from ten minutes prior to infection up to the time of infection. This led the group to concur that the effect of amantadine was on the initial infectious process. This

observation was further strengthened when adding amantadine late in the infection had no effect on the maturation of the virus and the release of the virus. This work also showed that in the presence of amantadine, influenza virus attached normally, but was prevented from penetrating the cell.

In 1969, Kato et al. reported that amantadine did not prevent virus penetration into the host cell, as previously suggested by Hoffmann (19). Instead, they showed that amantadine actually prevented uncoating of the virus after penetration into the cell. The method used in this study was a neutral red-labeled virus technique previously performed on poliovirus. It had been shown that incorporated neutral red causes the virus to be photosensitive, but this photosensitivity is lost shortly after the virus infects the host cell. Therefore, the loss of photosensitivity could be used as an indicator of uncoating. Kato and Eggers employed this technique with a fowl plague virus (FPV)-chick embryo cell system. Their results indicated that in untreated cultures, 90% of the virus that had adsorbed lost photosensitivity after one hour, indicating that almost all of the virus had uncoated. However, in treated cultures only 30% of the virus showed this loss after one hour, with no increase in this percentage after an additional two hours' incubation. They concluded that the virus did, in fact, penetrate the host cell in the presence of amantadine, but it was not uncoated.

In 1974, Dourmashkin and Tyrrell showed that influenza A virus penetrated the host cell in the presence of amantadine, and appeared in cytoplasmic vacuoles (7). Oxford et al. (25) claimed that they confirmed this, although the work was unpublished. Oxford also reported that his group expanded on these data and showed that in

amantadine-treated cultures using A/Hong Kong/1/68 (H3N2), penetration of the virus was normal, but polypeptide synthesis was inhibited. Additionally, RNA-dependent RNA polymerase activity was not affected.

A variety of biochemical analyses on FPV-infected chick embryo fibroblasts was conducted to determine more conclusively where in the infectious cycle amantadine works (31). First, Skehel et al. looked at its effect on protein synthesis. Like Oxford, they found that amantadine inhibited protein synthesis (50 ug/ml gave 50% inhibition; 200 ug/ml gave complete inhibition). They also showed that this inhibition was reversible; when amantadine was removed, viral protein synthesis resumed after a delay of approximately one hour. The study also found that when 200 µg/ml of amantadine was added before infection, viral mRNA synthesis was completely prevented. Again, this effect was reversible after removal of amantadine. They also reported that amantadine did not affect viral polymerase activity (which confirmed Oxford's work), nor did it inhibit the viral transcriptase.

Oxford first suggested the currently accepted method of action of amantadine in his review of 1980 (25). He states that amantadine and other amines are known to accumulate in lysosomes and tend to increase the pH in them. He cited one study by Ohkuma and Poole in 1978 that showed that ammonium chloride increased the pH of mouse peritoneal macrophages from 4.5 to 6.2, and amantadine followed suit by raising the pH from 4.5 to 5.5. Oxford also stated that fusion of the Semliki forest virus (SFV) membrane with that of lysosomes is very pH-dependent *in vitro*, and it was thought that it was this fusion that allowed the virus to move into the host cell cytoplasm. From these

data, Oxford put forth the possibility that it was this fusion mechanism that was inhibited by an amantadine-induced pH increase.

Use of Resistant Strains in Determining Mode of Action

In 1977, Appleyard reported the development of a method to use amantadine resistance as a genetic marker for influenza, simply by creating reassortant strains of viruses and determining their amantadine sensitivity or resistance (1). Up to this point, only hemagglutinin and neuraminidase were available as genetic markers. First he demonstrated the amantadine sensitivities of his experimental strains, showing that two strains were sensitive (A/Sing, BEL), two were intermediate (PR8, WSN) and one was resistant (Lee). Next, he created recombinant viruses by mixing A/Sing and a resistant strain of BEL. The result was a fairly high frequency of recombination -- at least 10% were mixed antigenically -- and about 17% of the viruses were amantadine resistant. The HA and NA mix showed no correlation to the acquisition of resistance. He then repeated a similar test using A/Sing and PR8. Again, a large percentage of the progeny A/Sing virus was resistant.

The next step in the study was to test to determine if amantadine sensitivity was transferable. For this, PR8 was mixed with A/Sing, and PR8 progeny were tested for sensitivity. A significant portion (18%) of the mixed PR8 exhibited sensitivity, leading to the conclusion that sensitivity, as well as resistance, could be transferred. Finally, Appleyard infected mice with mixed virus and was able to exhibit recombination and transfer of resistance. The final results indicated that resistance and sensitivity could be

transferred between influenza viruses, and that resistance was not connected to the hemagglutinin and the neuraminidase.

Finally in 1978, Lubeck et al. reported that they had pinpointed the gene responsible for conferring amantadine resistance in influenza viruses (24). In order to show this they expanded on Appleyard's work with recombinant strains. In this case, Lubeck et al. took it one step further by creating recombinants with the manipulation of all eight gene segments. First, using A/HK/8/68 (H3N2) and PR8, the group confirmed that resistance was not dependent on the parental HA and NA types. Then they created recombinants to study the influence of the genes coding for the NP, M, NS and P proteins. The results of this experiment showed that the M gene was the one that was associated with resistance to amantadine. These data were strengthened by performing a similar series of experiments with A/WSN/33 & A/NED/84/68 (H2N2) and WSN & HK viruses, all with the same results. This study also reported that the frequency of resistance was determined using HK virus. By passaging the virus in eggs, an estimated frequency of four resistant variants in 10,000 PFU was obtained.

The M2 Protein

Lamb et al. (1981) reported that the M gene coded for two proteins: the membrane protein M1, and a novel protein designated M2 (21). This work was done in response to reports in the influenza literature that the sequence of segment 7 of PR/8/34 had been determined and apparently there was a second open reading frame present that could code for 97 amino acids. In addition, this open reading frame was conserved in A/Udorn/72 a full 38 years later. This group showed in its report that indeed there was a

protein coded for in the second open reading frame, and this protein was translated from a separate mRNA. They also suggested that according to some additional data, the M2 mRNA had a leader sequence of 51 nucleotides that were identical to those in the M1 protein; this would seem to indicate that the start codon for the M2 mRNA is that of M1. Lamb also stated that this M2 protein had yet to be found in the influenza virions.

Lamb et al. further studied the M2 protein and showed that it is an integral membrane protein that is expressed on the influenza-infected cell surface (22). After homogenizing infected cells, the membrane fraction of the cells was analyzed and the result obtained showed that M2 copurified with the same membrane fractions as HA and NA. It was also reported that Triton X-100 and 0.5 M KCl were needed to solubilize M2, which is a characteristic of membrane proteins. These data, in addition to a hydropathy plot showing a prominent hydrophobic region, led to the conclusion that M2 is indeed an integral membrane protein. This study also proposed a model for the orientation of M2 in the membrane. After trypsinizing infected cells, it was found that part of the M2 protein was removed. However, an antibody to the COOH-terminal region could still precipitate the cut M2, while an amino-terminal antibody could not. This indicated that the amino-terminus projects from the cell membrane. After performing a series of more precise cuts, Lamb et al. commented that at least 18 amino-terminal residues are exposed. Finally, the study presented some samples of immunofluorescent staining using the amino-terminal antibody, which clearly showed that this portion of M2 did, indeed, project from the cell surface.

Several years later, the M2 protein was isolated in virions (36). This was facilitated by producing a high-titer monoclonal antibody that recognized a region at the amino-terminus of the protein. Purified virions were studied for the presence of M2 with the use of an immunoblotting technique that utilized the monoclonal antibody. By comparing the quantity of M2 versus that of other known influenza virion proteins, Zebedee et al. determined that M2 is represented at a very small number -- an average of 14-68 molecules per virion. This finding was confirmed with a (^{35}S)-cysteine labeling technique, and led to the conclusion that given the large number of M2 molecules expressed at the infected cell surface, the M2 protein is somehow selectively excluded from the progeny virus.

After much more work on the M2 protein, it was reported that its function is as an ion channel in the virion membrane (30). This was accomplished by expressing M2 mRNA in *Xenopus spp.* oocytes and performing a series of electrophysiological procedures. First, voltage clamping showed that hyperpolarization of the expressed M2 protein caused an inward current to occur, giving the first suggestion that the protein is indeed an ion channel. Addition of amantadine attenuated this current, which was effective in showing the target of the drug's action. Then, the case was made that ion channel activity could be further proven if M2 proteins expressed with mutations in the transmembrane domain showed different properties than wild-type. Two mutants were used: one with a three-amino acid deletion, and one with a valine addition and a substitution. The ion selectivity of these mutants were tested, and it was found that the deletion mutant had a greater permeability to Na^+ and the addition mutant had a greater

permeability to Cl^- . Since there were different permeabilities, it was concluded that the mutations must affect the pore-forming region of the channel. To test further the ion channel activity of the mutants, the group tested to see if the currents of the channels returned to resting after activation. Both mutants showed residual activation, with that of the addition mutant being greater. Also, the voltage dependence of activation differed between the two.

Since it appeared that M2 is indeed an ion channel, the next logical question to be asked was what activated it. Observation during the course of the experiments showed that unlike for the mutants, the wild-type M2 protein appeared to not be activated by a change in current; the current-voltage relationship was linear throughout the range of voltages used on the oocyte membranes, and the current amplitude did not change with time. A change in pH was studied, and it was found that by decreasing the pH from 7.4 to 5.4, the current amplitude increased dramatically. Further study showed that the wild-type M2 is permeable to sodium ions, but not really affected by chloride or potassium. Testing for hydrogen ion permeability was not performed. However, by all accounts, it appears that the M2 ion channel is activated by changes in pH.

M2 and Amantadine Resistance

This new protein, M2, was first suggested to be the cause of amantadine resistance by Hay et al. shortly after it was shown to be an integral membrane protein (10). The most important finding of this study involved the creation of amantadine-resistant isolates of Singapore, Rostock, and Weybridge by passaging them in the presence of amantadine. The M genes were sequenced and it was determined that in each

strain there was a single nucleotide substitution in the M2 genes. The amino acid substitutions were at residues 27, 30, 31, or 34, which are residues in the hydrophobic domain of the protein. These amino acid substitutions have been shown to occur consistently in amantadine-resistant human and avian strains, both *in vitro* and *in vivo* (9). The effect of HA type on resistance was discounted by sequencing those genes and finding no repetitious mutations that could be correlated with the mutations of M2.

While current evidence tells us that amantadine acts upon the M2 protein, it still is not known exactly how it binds and its molecular effect on the protein. Recent stoichiometric data shows that amantadine binds in a way consistent with a single drug molecule blocking the channel (34). It was also shown that the block is bidirectional. However, data from this same study indicate that the drug does not exhibit the behavior that would be expected if the drug actually bound to and blocked the pore of the M2 ion channel. This leads to the possibility that amantadine functions as an allosteric blocker by binding elsewhere near or on the protein and causing a conformational change to the channel.

Key to the acquisition of amantadine-resistance is the putative configuration of the transmembrane region of M2. This domain is made up of predominantly hydrophobic amino acid residues, with most natural isolates containing one polar (Ser31) and one charged (His37) residue. This has led to the consensus that this domain exists as an alpha-helix, with the hydrophobic faces toward the lipid bilayer of the virion envelope and the polar faces forming the ion channel. This configuration would place residues 27, 30,

31, and 34 at the polar face, suggesting that any amino acid substitution at these residues would naturally affect amantadine binding (32).

Amantadine Resistance in Clinical Isolates

While drug-resistant strains of influenza obtained *in vitro* have proven to be invaluable in studying the virus, such isolates occurring naturally *in vivo* are of concern. Amantadine resistance in clinical isolates was first reported in 1981 (13) when Heider et al. showed that two isolates from the 1980 Berlin epidemic were indeed resistant to amantadine and rimantadine. However, the origin of these isolates could not be traced, so the point of introduction of the resistant strains could not be definitively explained. According to this report, amantadine and rimantadine were not in use in Berlin at that time, so no apparent selective pressure was present. However, it cannot be ruled out that these isolates were imported from other countries, they were from Parkinson's patients on long-term amantadine therapy, or the resistance was due to the PR/8 influenza strain in the live vaccine used that year.

In 1986, Pemberton et al. (29) reported on a comprehensive study of H1N1 and H3N2 isolates to determine the occurrence of amantadine resistance in the general population. Three groups of isolates were used: one from a London epidemic in 1981/82, one from a school epidemic in the fall of 1980, and the third a mix of isolates from the UK, USSR and Japan over a period of 1977-1982. The results of the study showed that a range of resistances to low concentrations of amantadine was present in the isolates. Interestingly, there were several isolates that were not as susceptible to higher concentrations of amantadine as they were to the lower ones, although the low

concentrations of 2.5 mg/l are of more interest in a clinical setting (typical patient serum concentrations are approximately 1 mg/l). Another observation noted was that comparing chronologically arranged isolates showed an apparent increase in resistance. This strengthened the possibility that the clinical use of amantadine would select for resistant strains in the population.

More data on drug resistant strains of influenza A were presented in a report on the treatment of influenza with rimantadine in pediatric patients (4). Seven patients given rimantadine for treatment of influenza began to shed rimantadine-resistant virus 4-6 days after the start of treatment. After confirming that these isolates were resistant to rimantidine, Belshe et al. sequenced the M genes of the isolates and found two repeated nucleotide substitutions in the M2 open reading frame, as compared to the original sensitive isolates from the same patients. These nucleotide substitutions resulted in amino acid substitutions in the M2 proteins; two isolates exhibited the substitution Ala30Val, and five isolates had the substitution Ser31Asn. The latter substitution was exactly one of the substitutions described previously by Hay et al. in amantadine-resistant isolates (10). Belshe et al. took this experiment one step further than Hay's group; they passaged the original sensitive isolates *in vitro* in the presence of amantadine. The result was that the rimantadine-resistant isolates obtained in culture were similar to those found in the pediatric patients, indicating that studying drug resistance in cell cultures reflects the viral activity in humans.

While studying the use of rimantidine as post-exposure prophylaxis for influenza in the family setting, Hayden et al. (12) obtained several drug resistant isolates several

days after the initiation of treatment. Slightly more than half of the isolates were from index cases receiving rimantadine, whereas the remainder were from familial contacts (in this study, everyone in the family was given the same treatment: rimantadine or placebo). The suggestion was made that about half of the resistant virus cases in familial contacts were due to transmission directly from the index case. When gene 7 of each of the resistant isolates was sequenced, once again mutations were found that caused amino acid substitutions at residues 27, 30, or 31. Also reported was that one of the isolates was cross-resistant to amantadine. In light of the apparent transmission of drug-resistant virus, this group proposed that rimantadine is not appropriate for post-exposure treatment of influenza, unless the index case was left untreated.

To date, there has been only one report of an amantadine-resistant strain being isolated without any known drug pressure (18). This isolate was obtained during a study being conducted at three nursing homes, during which a total of five resistant isolates were obtained from separate patients. The other four isolates were recovered after the start of amantadine therapy. Sequencing of gene 7 of the isolate of interest showed that it did have a sequence that resulted in the common amino acid substitution, Ser31Asn, in the transmembrane region of the M2 protein. The other four resistant isolates exhibited this same mutation. Another interesting point about the novel resistant isolate was that the sequence of the M2 protein was homogeneous, which indicated that there was no evidence for a mixed infection of both resistant and sensitive strains. This is interesting, since previous studies have indicated that resistant strains seem to have a competitive

disadvantage against sensitive strains, and tend to emerge only after drug therapy has eliminated the sensitive strain.

Amantadine Resistant Strains versus Sensitive Strains

Currently the use of amantadine and rimantadine for treatment and/or prophylaxis of influenza A is usually limited to high-risk groups, such as pediatric patients and the elderly in nursing homes. However, there is concern that increased usage of the drug, especially during an epidemic or pandemic, could lead to the widespread generation of drug-resistant strains. Although this is certainly a real possibility, it is not known for sure if the resistant strains are biologically capable of making an impact on their own. Most of the resistant clinical isolates discussed seem to have a competitive disadvantage against sensitive strains, and tend to emerge only after drug therapy has eliminated the sensitive strain. To determine if resistant strains are competitive, studies have been undertaken to investigate their biologic characteristics versus sensitive strains, as well as to learn if the resistant strains are comparably transmissible, infective, and pathogenic.

A fairly comprehensive study was conducted using the A2/Singapore/1/57 influenza strain (drug-sensitive) and comparing it to a laboratory-derived resistant strain (26). Infections of eggs and monkey kidney cells showed that, at an undefined endpoint, the resistant strains exhibited hemagglutination (HA) titers, infectivity titers, and hemagglutination inhibition (HI) titers comparable to those of the sensitive strains. Further study showed that there were no differences detected in mean buoyant densities of the hemagglutinins, and electron microscopy showed no differences in size and morphology (27). When mice were infected with these strains, lung lesions and

consolidation were comparable in both types of infections. Virulence of drug-resistant strains was also tested in the ferret model (33), and again it was determined that there was no significant difference in virulence when they were compared to their sensitive counterparts.

Lavrov et al. (23) reported that they had obtained amantadine-resistant strains of A0WSN influenza. However, comparative testing with the drug-sensitive strain revealed no difference between the strains, except for the fact that at 56°C, the resistant isolate had an increased sensitivity, as evidenced by loss of HA activity.

In addition to being virulent, drug resistant strains appear to be adequately transmissible. Bean et al. (2), used the avian influenza virus A/chicken/Pennsylvania/1370/83 (H5N2) as a model to test for human influenza transmissibility. One group of chickens was infected with wild-type virus without amantadine, and another was infected and given the drug. Once several passages of chickens exposed to the treated group began shedding resistant virus, it was mixed with an untreated group. Then, several passages of uninfected chickens were exposed to the mixed group to see if the resistant strain could compete with the wild-type strain. The results were inconclusive after four tries: the contact birds twice shed only resistant virus, once shed only sensitive virus, and once shed both sensitive and resistant virus. Although the results do not give either strain a definite competitive advantage, it is apparent that the resistant virus is transmissible in a mixed strain setting.

Bean et al. then used some of the resistant isolates from the above experiments and tested them to determine if their virulence had been affected. Both resistant and

sensitive strains showed variable virulence as compared to the parental strain. They did show that one of the resistant isolates was still quite virulent in the presence of amantadine (it killed 4 of 6 birds), while birds that were infected with the wild-type strain survived with a drug concentration of four times lower. Finally, several of the resistant isolates were used to sequence gene segment 7. Not surprisingly, mutations were found that caused amino acid substitutions at residues 27, 30, and/or 31.

Another animal model in which the characteristics of amantadine-resistant versus sensitive strains were studied is that of the ferret (33). Known resistant isolates containing a common M2 protein single amino acid substitution (Val27Ala, Ala30Val, or Ser31Asn) were used to investigate the mutations' effect on virulence. Measures of virulence were level and degree of persistence of nasal infection, height and duration of pyrexia, level of nasal inflammatory cells, and level of lung infection. The results were very convincing; there was no significant difference in virulence when drug-resistant isolates were compared to their sensitive counterparts. These data led to the conclusion that the mutations in the M2 protein do nothing to "attenuate or potentiate" the virulence of human influenza virus.

On a molecular level, it has been shown that several influenza strains with natural "resistance" mutations exhibit interesting M2 protein ion channel characteristics (30). When measuring the current through the ion channels in resistant strains versus wild-type strains, Pinto et al. discovered that the resistant mutants (except for one) had currents with larger amplitudes than in wild-type. More interesting yet, when amantadine was

added, these currents were not significantly attenuated, as in wild-type strains, and for one mutation (A30T) the amplitude was greater.

Objectives

The primary goal of this project is to investigate the possibility that drug-resistant strains of influenza A virus (H3N2) exhibit significant differences in replication rate as compared to drug-sensitive strains. While experimentation has shown that in many characteristics drug-resistant strains are comparable with sensitive strains, it still remains to be determined why resistant strains appear only after selection by drug pressure.

This inability to compete is highlighted by the fact that *in vitro*, when these drug-resistant strains were mixed with sensitive strains and passaged, only sensitive isolates were recovered (16). Another study (33) has shown in the ferret model that nasal virus titers were comparable between drug-resistant and -sensitive strains (with sampling occurring every ten hours), which implies that replication time for the strains is about the same. This conclusion will be tested in an *in vitro* system, with frequent sampling, in order to determine the kinetics of the growth curves of both drug-resistant and -sensitive strains. This technique will also be used to investigate a secondary objective of this study: to determine if the presence of amantadine HCl alters the growth kinetics of drug-resistant influenza strains in such a way as to give them a competitive advantage over sensitive strains.

MATERIALS AND METHODS

Cell Line and Virus Stock

Cell line. Madin-Darby canine kidney (MDCK) cells were used for replicating influenza virus. Cells were grown in minimal essential medium with Earle's balanced salt solution [MEM(e); Flow Laboratories, Inc., McLean, VA; GIBCO Laboratories, Grand Island, NY) supplemented with 7.5% fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, UT), 2 mM l-glutamine (Sigma Chemical Company, St Louis, MO), and a combination of 100µg/ml streptomycin and 100 U/ml penicillin (GIBCO). The pH of the medium was adjusted to approximately 7.4 with 7.5% sodium bicarbonate or carbon dioxide gas. Cells were grown in either Costar (Cambridge, MA) or Corning (Corning Glass Works, Corning, NY) tissue culture vessels and were replenished with fresh medium every 4-5 days. Stock cultures were incubated at approximately 35°C in an atmosphere containing 5% carbon dioxide.

Cells were subcultured every 7-21 days with a split ratio of 1:6. Cells were passaged using a trypsin versene solution (ATV) consisting of 0.4% w/v trypsin (Difco Laboratories, Detroit, MI) and 0.1% w/v EDTA (Sigma) in phosphate buffered saline (PBS: 8.0 g/l NaCl, 0.3 g/l KCl, 0.073 g/l Na₂HPO₄, and 0.02 g/l KH₂PO₄). ATV was used to wash the cells for approximately 30 seconds, then was aspirated. A second volume of ATV was left on the cells for 5-10 minutes to dislodge the cells from the plasticware. After cells were in solution, they were diluted in the desired amount of MEM(e) and aliquotted to new vessels. MDCK cells between passages 70 and 80 were used for this study. Cell stocks were tested periodically for contamination with the use of

antibiotic-free medium and the Hoechst stain (Sigma), a fluorescent stain used to detect mycoplasma DNA (5).

Virus cultures. The viruses used in these experiments, influenza A (H3N2), were a generous gift of Drs. Robert Atmar and Janet England of the Baylor College of Medicine. These strains were isolated from an immunocompromised patient and were screened at Baylor for their rimantadine sensitivity or resistance. Resistant isolates were further tested by the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, for the specific M2 protein mutation causing resistance (Table 1).

TABLE 1. Influenza A (H3N2) isolates used.

Isolate Number	Patient Treatment	Screen Results ^a	Confirmation ^b	M2 Mutation
65301	None	Sensitive	-	-
65634	Rimantidine	Resistant	Resistant	Ser31Asn
66041	Rimantidine	Resistant	Resistant	Ala30Val

^a Performed at Baylor College of Medicine

^b Performed at CDC

Plaque Purification

Virus isolates were plaque purified using a modified single overlay plaquing procedure (25). MDCK cell monolayers (3-4 days old) in 35 mm² diameter 6-well plates were washed two times with Hanks' balanced salt solution (HBSS; Sigma) buffered with 0.35g/L sodium bicarbonate prior to application of drug or virus. Wells in which drug

resistant virus was to be inoculated were incubated for 15 minutes with 0.5 ml HBSS containing 2 µg/ml amantadine hydrochloride (amantadine; Sigma; lot #115H3700). Monolayers were inoculated with 0.1 and 0.01 µl of the desired virus in 0.5 ml of HBSS and incubated at 35°C for 30-60 minutes to allow the virus to adsorb to the cells. After adsorption, excess liquid was aspirated and the monolayers were overlaid with 2 ml of a 1:1 mixture of 1.8% agarose (BBL, Div. Of Becton, Dickinson & Company, Cockeysville, MD) and MEM(e) supplemented with 0.15% w/v bovine serum albumin (Sigma), 0.5 u/ml TPCK trypsin (Cooper Biomedical), 100 µg/ml DEAE-dextran (Sigma), and a combination of 100 µg/ml streptomycin and 100 U/ml penicillin (GIBCO). For studies of drug resistance, amantadine (2 µg/ml) was added to the overlay. The overlay was allowed to solidify and the plates were incubated until plaque formation was noted, usually 3-4 days. The plaques were visualized by staining with 0.7 ml of 0.025% neutral red (Sigma) in PBS and incubating for two hours (8). Extra stain was aspirated.

Virus from individual plaques were transferred to MDCK monolayers in 12-well tissue culture plates by scraping the edges of a plaque area under the agar overlay with a sterile pasteur pipette and aspirating the sample into the medium covering the monolayers. The medium used for infection consisted of MEM(e), 2 mM l-glutamine, 0.5u/ml TPCK trypsin (Worthington Biochemical Corp., Freehold, NJ), and antibiotics ("MEM(e) plus trypsin"). Plates were incubated until maximum cytopathic effect (CPE) was obtained. The medium containing virus was then harvested to use as viral stock.

Quantification of Virus - Viral Titration

Ten-fold (log) serial dilutions of the virus were performed in either 12-well plates or 96-well microtiter plates. Monolayers in 12-well plates were washed twice with HBSS, with the final wash discarded. Serial ten-fold dilutions were performed by diluting 50 μ l of virus in 0.45 ml of HBSS in Wheaton vials. Each dilution was transferred to three wells, inoculating 0.1 ml per well. The virus was allowed to adsorb 30-45 minutes, followed by the addition of 1.5 ml of MEM(e) plus trypsin to each well. Presence or absence of cytopathic effect (CPE) was recorded every 24 hours until the results showed no change for two consecutive readings (3-4 days). Virus titer was calculated from the CPE results using the Reed-Muench method and reported as the tissue culture infectious dose affecting 50% of the cultures (\log_{10} TCID₅₀) (28).

For titration in the 96-well microtiter plates, 50 μ l of virus was diluted in 0.45 ml of MEM(e) plus trypsin in ten-fold dilutions. Each dilution was transferred to a microtiter plate in quadruplicate, with 0.1 ml of the inoculum per well. The plates were incubated at 35°C for four days and examined for CPE. The virus titer was calculated using the Reed-Muench method.

Amantadine Cytotoxicity Study

This study was performed to determine at which amantadine concentration, if any, cytotoxicity occurred in MDCK cells. Monolayers in 12-well plates were washed two times with HBSS, then inoculated with MEM(e) plus trypsin containing concentrations of

amantadine varying from 0.1 µg/ml to 50 µg/ml. Cells were incubated for 48 hours and any signs of cell destruction were noted.

Amantadine Sensitivity Screen

Virus stocks were tested to confirm amantadine sensitivity or resistance using a modification of the 96-well microtiter plate titration method described above. Virus was diluted in MEM(e) plus trypsin in ten-fold dilutions. In addition, each virus was also diluted in MEM(e) plus trypsin with 5 µg/ml of amantadine. Each dilution was transferred to a microtiter plate in quadruplicate, with 0.1 ml of the inoculum per well. The plates were incubated at 35°C for 30 hours and examined for CPE. The virus titer was calculated using the Reed-Muench method. Viruses exhibiting a 10-fold or greater reduction in titer due to the addition of amantadine were considered to be sensitive.

Virus Replication Experiments

Monolayer growth. Four milliliter Wheaton vials were inoculated with 0.5 ml of MDCK cells suspended in MEM(e) containing approximately 2×10^6 cells. The vials were incubated at 35°C for 2-3 days until monolayers were confluent. Cells from three uninoculated vials were used to determine the average number of cells per vial.

Cell counts. The growth medium was aspirated from each vial and the monolayer was washed once with 0.2 ml of ATV. Another 0.2 ml of ATV was added to each vial and removed after washing for approximately 30 seconds. The vials were incubated five minutes to loosen the cells from the glass. After incubation, the cells were suspended in 0.5 ml PBS and centrifuged at $3,000 \times g$ for 10 minutes. The PBS supernatant was

removed by aspiration and the cell pellets were washed with PBS a second time. After the second wash, the cell pellets were resuspended in 0.5 ml PBS and 0.1 ml of each of the cell suspensions were diluted in 20 ml of PBS. Total cell counts were conducted using a Coulter counter (Model ZB1).

Virus growth curve. Prior to infection, cell monolayers were washed two times with HBSS. The cells were inoculated with virus in duplicate at a multiplicity of infection of approximately 1 virion/cell (MOI=1). The infected cells were incubated at 35°C for 45 minutes to allow the virus to adsorb to the cells. After incubation, unattached virus was removed by aspiration and the monolayers were washed three times with HBSS. After the third wash, 0.5 ml of MEM(e) plus trypsin was added to each vial and infected cells were incubated at 35°C for up to 30 hours. Samples were taken at 0, 2, 4, 6, 8, 10, 12, 14, 16, 20, 24, and 30 hours. For quantification of virus, each sample was frozen and thawed three times and centrifuged for ten minutes at 3,000 x g to remove cell debris. Virus titers of each sample were obtained using the 96-well microtiter plate titration method described previously.

Growth curve with amantadine. Virus growth curves in the presence of amantadine were conducted as described above with modifications. Prior to inoculation with virus, HBSS with 5 µg/ml of amantadine was added to each vial for 30 minutes. Virus was then inoculated onto the monolayers and allowed to adsorb 45 minutes. Unattached virus was removed by washes with HBSS, infection medium with 5 µg/ml of amantadine added, and cultures incubated and sampled periodically as described above.

RESULTS

Cell Culture Quality Control

The MDCK cell line used in this study maintained confluency and normal cellular morphology when grown in antibiotic-free medium, and no contaminating organisms were recovered. Hoechst stain preparations revealed no evidence of mycoplasma infection.

Quality Control of Replication Studies

Quality control for each growth curve included controls containing only media collected at 0 and 30 hours. In all cases, MDCK cell monolayers inoculated with these supernatants remained confluent and healthy, indicating that the media did not interfere with virus growth. Also included in each growth curve was a virus control, collected at 30 hours, that did not undergo washing after inoculation and adsorption. This control was included so that in the event that an isolate did not replicate during the course of 30 hours, it could be determined if the washing step hindered virus growth.

Two additional types of controls were added to those growth curves in which amantadine was added. Media controls containing 5 µg/ml of amantadine were collected at 0 and 30 hours to ensure that the addition of drug did not affect cell growth. Also, a control drug-sensitive isolate was inoculated onto monolayers with and without amantadine. These samples were collected at 30 hours, and a reduction in virus titer in the supernatants was used as an indication that the amantadine used was effective.

Media controls with amantadine showed no evidence of causing any cytopathic effect (CPE) on the MDCK cell monolayers. Therefore, it can be concluded that any

CPE detected upon titration of the growth curve samples was due to the replication of the isolates. For the growth curves including amantadine, the sensitive virus controls showed a reduction in titer due to the drug for all growth curves, with the exception of one experiment involving 65634-P3a. As a result, only two curves with the addition of amantadine will be discussed for this isolate.

Amantadine Cytotoxicity Study

Monolayers grown in amantadine concentrations of 0.1 $\mu\text{g/ml}$ to 1.0 $\mu\text{g/ml}$ showed no signs of cytotoxicity. The monolayers maintained confluency, and cellular morphology was consistent with that of monolayers grown in media without the drug. Concentrations of 2.5 and 5.0 $\mu\text{g/ml}$ caused a small increase in the numbers of rounded and refractory cells, indicating a small degree of cell damage. However, the overall appearance of the monolayers in these drug concentrations paralleled that of the negative control monolayers. Amantadine concentrations of 10 to 50 $\mu\text{g/ml}$ resulted in more prominent signs of cytotoxicity, as evidenced by large clumps of dead cells, rounded cells on the well periphery, and some missing portions of the monolayers. These results led to the conclusion that amantadine concentrations of 5.0 $\mu\text{g/ml}$ or less were suitable for experimentation.

Amantadine Sensitivity Screen

The original sample of isolate 65301 (drug-sensitive) supplied by BCM was tested in triplicate to determine an average reduction in titer due to 5 $\mu\text{g/ml}$ of amantadine. This reduction in titer was calculated by dividing the virus stock titer obtained in medium

without drug by the virus titer obtained in the presence of drug. An average 10-fold reduction in titer at 30 hours post-infection was achieved, and this reduction was used as a threshold to determine sensitivity versus resistance. Each virus stock used in virus growth experiments was tested to confirm that the samples retained their sensitivity or resistance to amantadine (Table 2). All stocks of 65301 were confirmed to be amantadine-sensitive and all stocks of 66041 and 65634 were shown to be resistant.

Virus Replication Experiments

Virus isolates. To determine if amantadine-resistant influenza isolates exhibited a replication disadvantage as compared to sensitive isolates, 30-hour growth curves were generated for one sensitive isolate (65301-P3a) and two resistant isolates (66041-P3a and 65634-P3a). These virus isolates were collected during the third plaque passage of the respective original samples received from BCM.

Generation of growth curves. Growth curves for each virus consisted of duplicate trials at each time point, with a total of three separate growth curves conducted for each isolate. The virus titer of each test replicate was determined by titrating the supernatant of the frozen/thawed samples and calculating the \log_{10} TCID₅₀/ml using the Reed-Muench method. The titers of both samples at the separate time points were averaged and plotted to generate a graphical growth curve. One representative plot for each virus isolate tested is shown (Fig. 1). Also shown, for visual comparison, are the average growth curves for each isolate. These curves were generated by averaging the viral titers at each time point for three growth experiments per virus isolate (Fig. 2).

TABLE 2. Results of amantadine sensitivity testing of virus stocks at 30 hours post-infection^a.

Virus Stock Represented	Sample Tested	Viral Titer in 0 µg/ml (log ₁₀ TCID ₅₀ /ml)	Viral Titer in 5 µg/ml (log ₁₀ TCID ₅₀ /ml)
65301-P3a ^b (S1 to S10)	65301-P3a-S3	3.50	2.33
65301-P3a (S11 to S20)	65301-P3a-S12	2.50	No growth
65634-P3a (S1 to S10)	65634-P3a-S4	4.00	3.67
65634-P3a (S11 to S20)	65634-P3a-S13	4.00	4.00
65634-P3a (S21 to S32)	65634-P3a-S32	2.00	1.67
65634-P3a (S33 to S43)	unaliquotted stock	3.67	3.67
66041-P3a (S1 to S10)	66041-P3a-S1	4.60	4.67
66041-P3a (S11 to S20)	66041-P3a-S12	4.00	5.00

^a Each stock tested twice (except 65634-P3a-S21 to S32); replicate results not shown.

^b "P3" indicates the passage during plaque purification in which the isolate was collected; "a" denotes it is the first sample collected within a passage.

^c "S#" designates the number of aliquots collected for a given batch of virus stock grown.

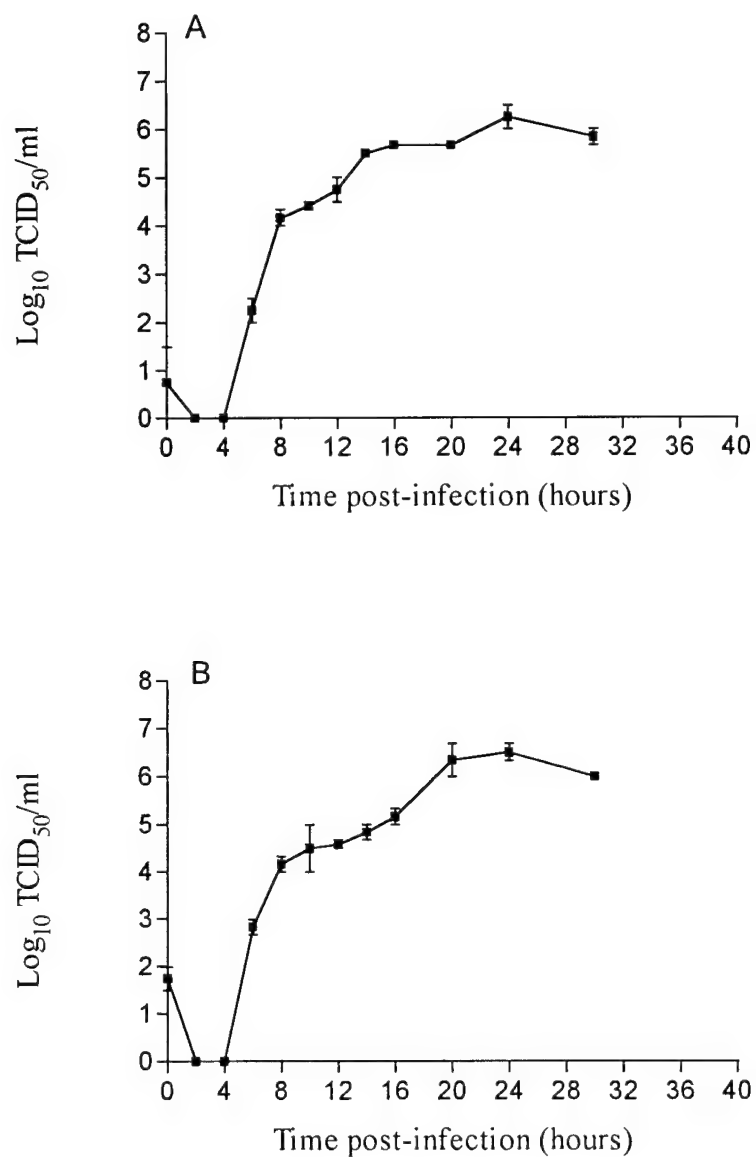


FIG. 1. Representative 30-hour growth curves. Isolate 65301-P3a (A) is amantadine-sensitive; 66041-P3a (B) and 65634-P3a (C) are resistant. Multiplicity of infection (MOI) was approximately 1.0, with the exception of 65301-P3a (MOI \approx 2.0).

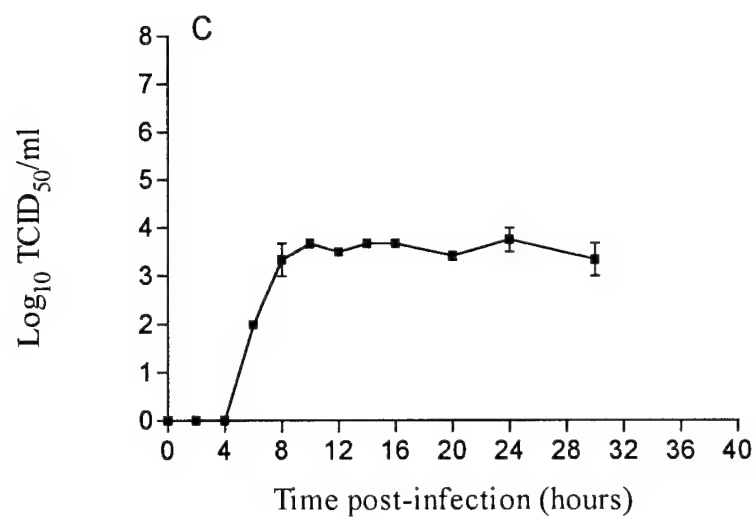


FIG. 1. Continued.

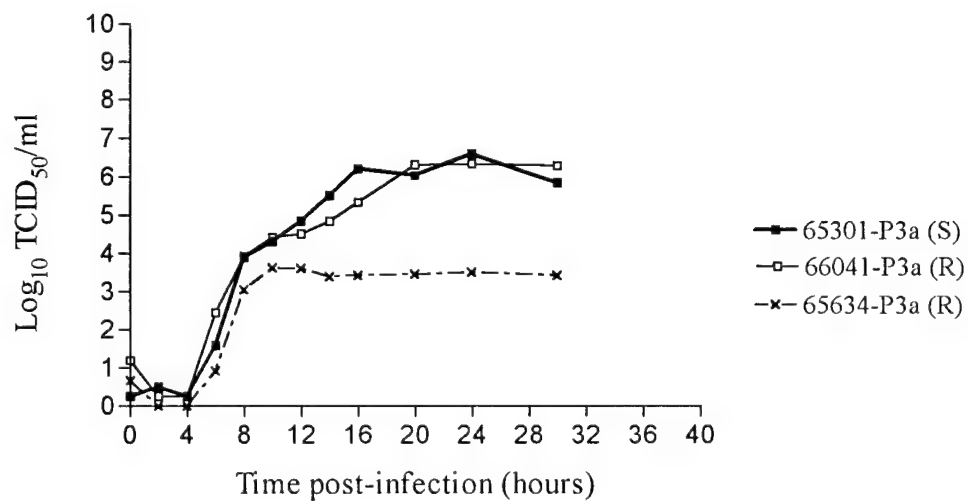


FIG. 2. Average growth curves. These curves were generated by averaging the viral titers at each time point for three growth experiments per virus isolate.

Analysis of growth curves. Numerical analysis of the growth curves was accomplished to augment visual comparison of the curves. In order to compare the replication characteristics of each isolate, linear regression analysis was performed on the growth phase of each curve. The slope of the resulting best-fit line was used as a measure of the increase in virus progeny per unit of time (in this case, per hour). In addition, the "carrying capacity" of each virus isolate was visually estimated for each curve. The carrying capacity is the titer at which the maximum numbers of progeny virus have been produced and the population is maintained at a relatively constant numeric level. Representative graphs showing linear regression analysis and estimated carrying capacity are shown (Fig. 3). The numerical results of these analyses (Table 3) show that resistant strains do vary in their replication characteristics. The isolate 66041-P3a had a higher virus yield than did 65634-P3a, but it had a smaller population growth rate. The sensitive strain used for comparison, 65301-P3a, had replication characteristics that more closely resembled those of 66041-P3a. A second sensitive isolate that was tested exhibited replication characteristics much like those of 65634-P3a (data not shown), indicating that this variability in growth exists both in sensitive and resistant strain populations.

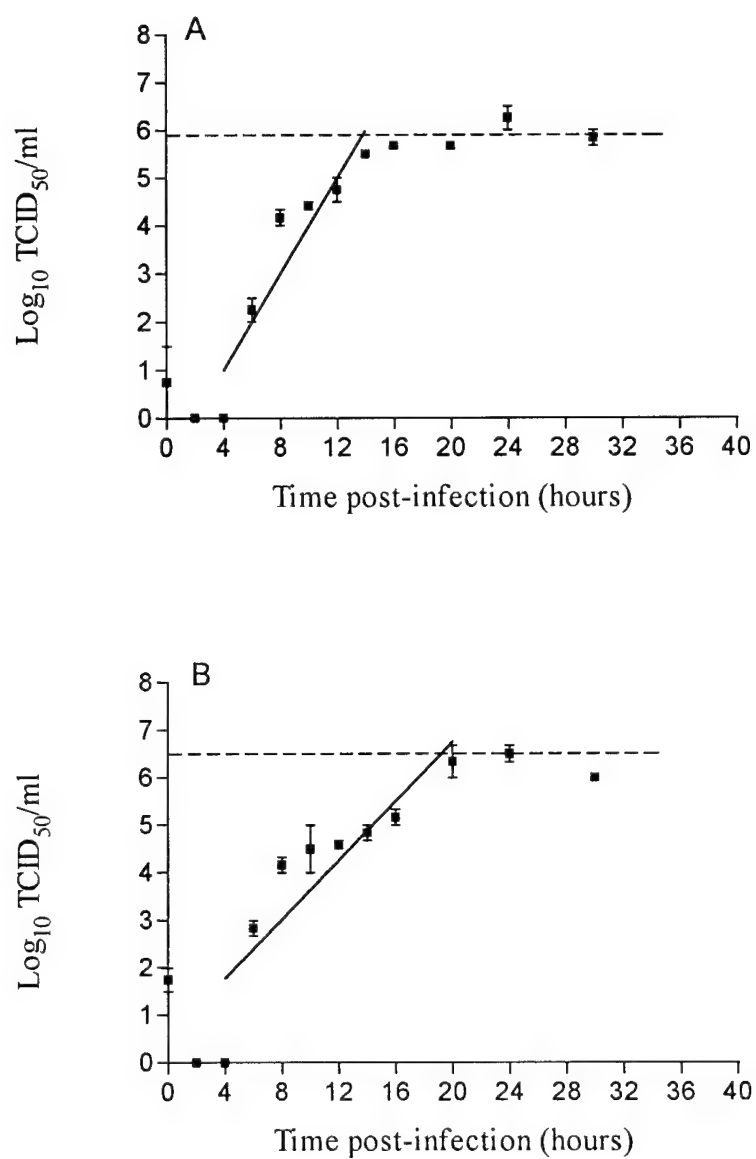


FIG. 3. Representative 30-hour growth curves with numerical analysis. Solid lines indicate linear regression of the growth phase of the curves, while dashed lines show estimated carrying capacity. Isolate 65301-P3a (A) is sensitive; isolates 66041-P3a (B) and 65634-P3a (C) are resistant.

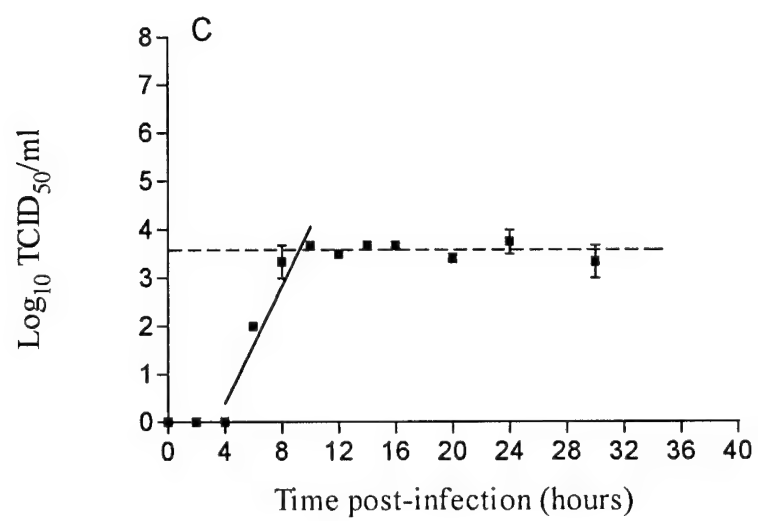


FIG. 3. Continued.

TABLE 3. Numerical analysis of 30-hour growth curves.

Isolate	Growth curve replicate	Multiplicity of infection (virions/cell)	Slope of regression line	Carrying capacity (\log_{10} TCID ₅₀ /ml)	Average carrying capacity (\log_{10} TCID ₅₀ /ml)
65301-P3a (sensitive)	1	0.80	0.583	6.50	6.20
	2	2.50	0.486	6.30	
	3	2.10	0.504	5.80	
66041-P3a (resistant)	1	1.00	0.314	6.45	6.28
	2	1.00	0.397	5.20	
	3	0.97	0.383	7.50	
65634-P3a (resistant)	1	1.00	0.617	3.50	3.46
	2	1.00	0.729	3.50	
	3	1.00	0.542	3.40	
66041-P3a + ^a	1	1.00	0.360	4.50	5.73
	2	0.97	0.329	6.20	
	3	0.97	0.326	6.50	
65634-P3a +	1	0.97	0.663	3.40	3.80
	2	1.00	0.428	4.20	

^a Denotes growth studies in the presence of 5 μ g/ml of amantadine.

Replication studies with amantadine. In order to determine if amantadine alters the growth characteristics of drug-resistant viruses, growth curves with the addition of 5 $\mu\text{g/ml}$ of amantadine for isolates 65634-P3a and 66041-P3a were conducted. As for the growth curves generated without the addition of drug, analysis of these curves included linear regression of the growth phase and determination of the carrying capacity (Table 3). Average growth curves of the resistant isolates, with amantadine versus without amantadine (Fig. 4) and representative curves with numerical analysis are shown (Fig. 5). These data indicate that the replication of drug-resistant strains was not significantly changed in the presence of amantadine.

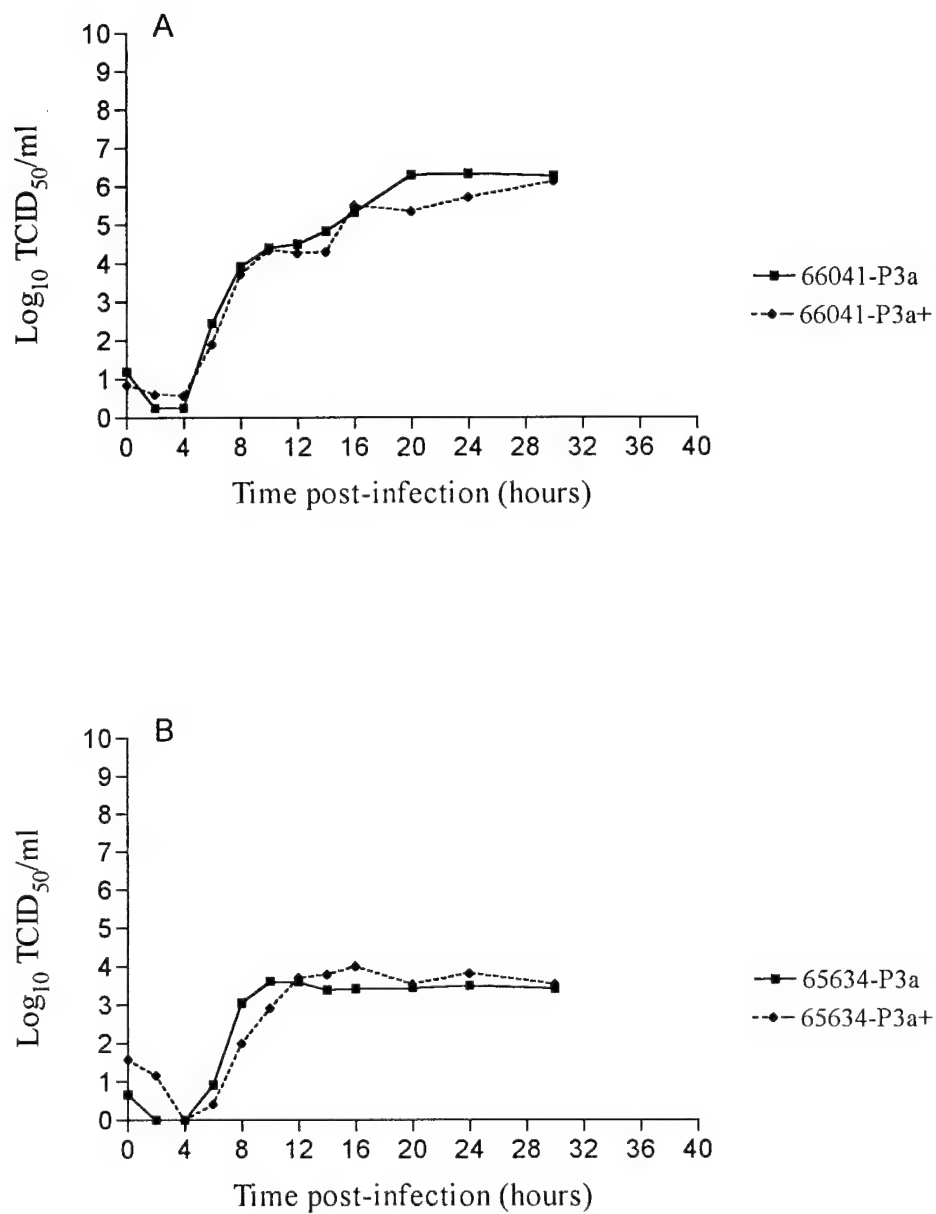


FIG. 4. Average growth curves of drug-resistant isolates, with and without amantadine. The "+" added to an isolate number in the legends denotes addition of amantadine.

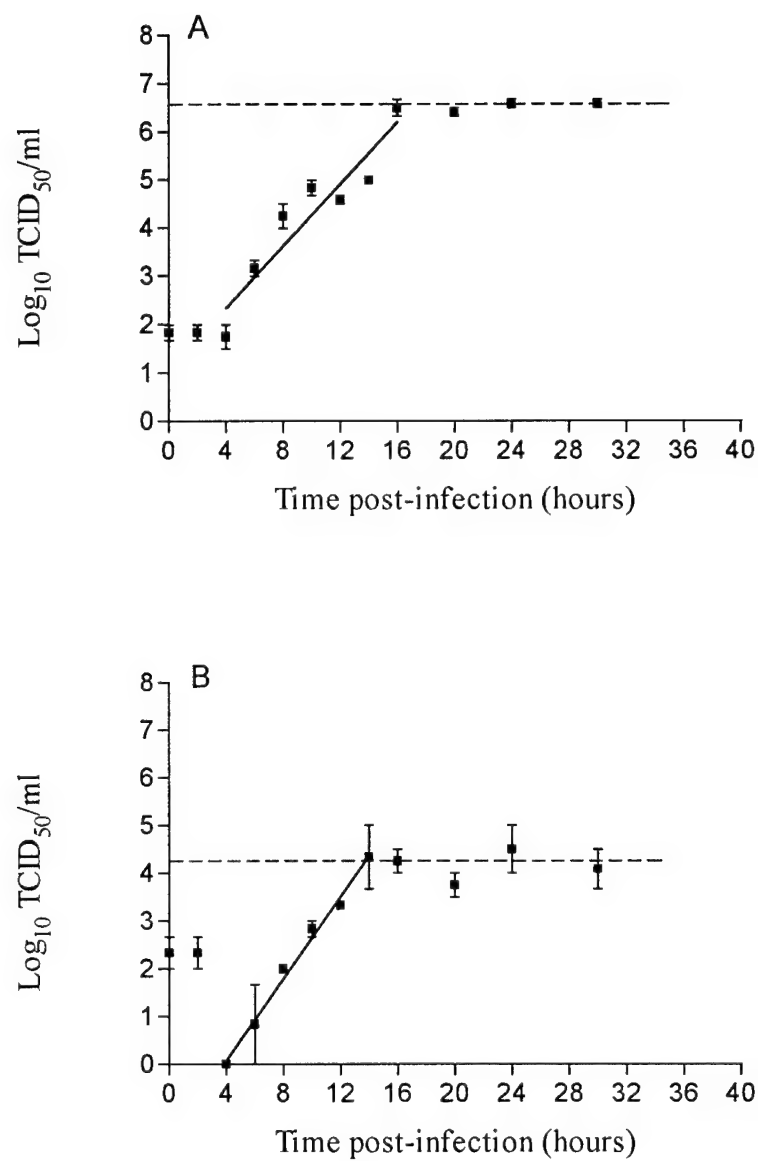


FIG. 5. Representative growth curves with addition of 5 $\mu\text{g}/\text{ml}$ of amantadine. Solid line indicates linear regression of the growth phase of the curves, while dashed lines show estimated carrying capacity. Isolates are: 66041-P3a+ (A) and 65634-P3a+ (B).

SUMMARY AND CONCLUSIONS

Since the first report of amantadine-resistant strains of influenza in 1981 (13), there has been much speculation about the epidemiological impact of these strains should the use of amantadine and rimantadine become widespread during an influenza pandemic. To date, there have been only two reports made of confirmed resistant strains that were isolated from patients with no drug treatment (11). All other resistant strains have been collected only after therapy with either rimantadine or amantadine.

Because naturally-occurring resistant strains are not isolated more often, it was concluded that the drug-resistant phenotype does not confer any type of selective or replicative advantage over the sensitive phenotype (11). As a corollary, the suggestion was made that those viruses with the susceptible phenotype may have a biological advantage over their resistant counterparts (3). This study was conducted to study growth characteristics of sensitive and resistant isolates to see if, indeed, one phenotype has a replicative advantage over the other.

The virus isolates chosen for this study were collected during a period of illness from an immunocompromised patient with confirmed influenza (H3N2) infection. Because the sensitive and both resistant isolates (each with a different mutation) were from the same patient, we concluded that comparing the replication characteristics of these isolates would provide a model for naturally-occurring influenza infections. The emergence of different drug-resistant mutations in immunocompromised patients has been documented, and it is thought that this genetic variability reflects the heterogeneity of infecting virus populations (20).

Visual comparison of the 30-hour average growth curves provided the first indication that infecting influenza populations are indeed heterogeneous (Fig. 2). It is apparent that two distinct populations of virus are present: a "low-titer" population, with carrying capacity titers of less than $5 \log_{10}$ TCID₅₀/ml, and a "high-titer" population exhibiting maximum titers well above $6 \log_{10}$ TCID₅₀/ml. More striking is the fact that drug susceptibility appears to have no effect on these growth characteristics. Isolate 65634-P3a is a "low-titer" virus, while 65301-P3a and 66041-P3a are "high-titer" viruses. These data lead to the preliminary speculation that drug-resistant viruses can indeed compete with drug-sensitive viruses, *in the case of "high-titer" isolates*. However, it appears that viruses much like the 65634-P3a isolate may not achieve viral titers high enough to outcompete isolates such as 65301-P3a.

In order to compare the isolates in a more quantitative manner, linear regression analysis was performed on the growth phase of each of the multiple curves generated per virus. Because the slopes of the best-fit lines are a measure of the increase of the virus populations per hour, they can be used to compare the replication rates of the individual isolates.

The amantadine-sensitive isolate 65301-P3a had an average slope of $0.524 \log_{10}$ TCID₅₀/ml per hour. The resistant isolate 66041-P3a exhibited a significantly different ($p < 0.001$) average slope of $0.365 \log_{10}$ TCID₅₀/ml per hour. Using visual comparison, one would anticipate the slopes to be approximately the same, especially since both isolates reach statistically the same carrying capacities. However, 66041-P3a reaches its

carrying capacity four hours later than does 65301-P3a, explaining the slightly lower population growth rate.

The average replication rate of $0.629 \log_{10} \text{TCID}_{50}/\text{ml}$ per hour for 65634-P3a was surprising. With this higher rate, it would be expected that this isolate should reach much higher titers than those of the two other isolates. Closer inspection of the average growth curves in Fig. 2 reveals an important feature -- 65634-P3a reached its carrying capacity at approximately 10 hours. This is 6-10 hours before 66041-P3a isolates achieved maximum titers.

Because the majority of resistant virus isolates emerge only after drug therapy, it is natural to question the basis for this occurrence. The most obvious explanation is that titers of sensitive strains are reduced, allowing resistant strains to become the predominant viruses in an infection. In effect, resistant strains are selected for under drug pressure. However, there may be other contributing factors to the emergence of resistant isolates.

Since amantadine (and rimantadine) acts by entering infected, and potentially infected, cells and interfering with the ion channel of the M2 protein of the endocytosed virion, it in effect changes the internal environment of the host cell. This leads to the speculation that not only can resistant strains withstand drug therapy, but also this same therapy enhances resistant strain growth in some manner. While plaque purifying and harvesting the resistant isolates 66041 and 65634 under drug pressure, the incidental observation was made that these isolates seemed to thrive better than their counterparts grown without the benefit of amantadine. This was evidenced by qualitative observations

such as cytopathic effect on infected monolayers was more widespread, and complete destruction of monolayers tended to occur earlier. Therefore, growth experiments with the addition of amantadine were performed to test the hypothesis that drug therapy does enhance resistant strain growth.

Visual comparison of the 30-hour average growth curves of the resistant isolates in the presence and absence of amantadine provided the first indication that drug therapy did not enhance, or inhibit, the growth of the virus isolates (Fig. 4). In the case of 66041-P3a, the growth characteristics are almost identical. The average growth pattern of 65634-P3a in amantadine indicates that a slightly higher carrying capacity may be reached, but it does not appear to be significant.

To confirm this conclusion, numerical analyses described previously were performed on each of the curves generated per virus in the presence of amantadine. For both 66041-P3a and 65634-P3a, amantadine did not significantly increase or decrease ($p>0.05$) the change in virus population during the growth phase as compared to virus grown without drug. This is also the case for the average carrying capacity of the isolates.

Although a very limited number of isolates were tested, several preliminary conclusions can be made about the composition of naturally-occurring influenza infections. The first observation is the heterogeneity of the population. The results presented indicate that different clones isolated from a patient exhibit very different growth characteristics: some grow to high titers while others are not quite as successful in producing such large numbers of progeny.

This same heterogeneity exists not only in the population as a whole, but within the categories of sensitive and resistant isolates as well. For instance, choosing random virus clones to test from the 65301 sample resulted in both a high-titer isolate and a low-titer isolate (data not shown). While multiple clones were not tested for the 66041 and 65634 samples, one could predict that more comprehensive testing would yield a mix of isolates much like that of the 65301 sample.

Because the resistant isolates did show such a variability in growth characteristics, it is possible to more firmly substantiate Hayden's suggestion that resistance does not confer any selective advantage to a virus strain. Conversely, sensitivity alone does not appear to give virus isolates any type of advantage, either. Therefore, the possibility exists that drug-resistant strains are not isolated until after drug therapy simply because the numbers of these viruses are small compared to those of drug-sensitive viruses.

For example, the 65634 isolate contains the mutation Ser31Asn in the M2 protein, which is the most common mutation reported (11). This implies that resistant strains with this specific substitution are more numerous in natural populations as compared to strains with other mutations. If this is indeed the case, and if the clone tested in these experiments is representative of most virus strains containing this mutation, the possibility exists that these strains just cannot achieve the virus titers necessary to be detected when combined with "high-titer" virus strains, such as 65301-P3a. Only when amantadine or rimantadine are used and the titers of drug-sensitive strains are reduced can these drug-resistant strains be detected as a major portion of the population.

This scenario is only one of many that can be devised when mixing high- and low-titer viruses, drug-resistant and -sensitive viruses. In order to more firmly establish the general character of these populations, the types of growth experiments and analyses presented here must be performed on a larger number of the virus clones collected from each of the respective samples 65301, 65634, and 66041.

The data presented also provide strong evidence that drug therapy does not itself enhance or diminish the replication of drug-resistant strains. Again, since only a few isolates were tested in this study, a more comprehensive sampling of resistant clones is necessary to better substantiate this conclusion. Because addition of the drug did not significantly increase virus replication, this study does provide more evidence for the suggestion that the emergence of resistant strains during drug therapy is due to the selection against sensitive strains .

Other investigators have shown that resistant strains are just as transmissible and pathogenic as sensitive strains (2), and, as Sweet et al. (33) suggest, these experiments show that certain resistant strains can compete with comparable virus titers. Therefore, the question remains -- will resistant strains be a significant problem during widespread use of drug therapy during a pandemic? These data presented in this study provide strong evidence that drug-resistant strains do, indeed, possess the potential to be a major public health problem.

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VITA

NAME: Carol Christine Walters, Capt, USAF, BSC

SPECIALTY: Biomedical Laboratory Officer/M043T3A

ADDRESS: 382d TRS/XYAC
917 Missile Road Ste 3
Sheppard AFB TX 76311-2263

EDUCATION AND TRAINING:

Aug 97 MS, Medical Sciences
Texas A&M University
College Station, TX

Aug 92 USAF School of Medical Technology
Andrews AFB MD

Dec 89 BS, Microbiology
Texas A&M University
College Station, TX

PROFESSIONAL ORGANIZATIONS:

Society of Armed Forces Medical Laboratory Scientists
American Society of Clinical Pathologists (MT)
National Certification Agency (CLS)

PROFESSIONAL HONORS:

Aug 97 Summa cum laude, Medical Sciences
Texas A&M University

Feb 95 Company Grade Officer of the Year, 1994
Tinker AFB OK

Aug 92 Distinguished Graduate
USAF School of Medical Technology

Dec 89 Cum laude, Microbiology
Texas A&M University